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Genetic Diversity and Species Relationship of Some Terrestrial Bladderwort (*Utricularia* L.) as Revealed by Inter Simple Sequence Repeat (ISSR) Markers

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Genetic diversity and species relationships among ten species of *Utricularia* were investigated using inter simple sequence repeat (ISSR) markers. Six primers generated 236 ISSR fragments across the ten species with an average of 39.3 fragments per primer. *Utricularia longifolia* presented the highest number of fragments whereas *U. amethystina, U. humboldtii and U. uliginosa* provided the least. The highest similarity was detected between *U. alpina* and *U. longifolia* among the species studied while the highest pairwise genetic distance was found between *U. caerulea* and *U. longifolia*. Principal coordinate analysis was conducted to view the clustering pattern of the taxa which revealed that *U. alpina* and *U. longifolia* clustered together whereas *U. amethystina, U. bifida, U. caerulea, U. calycifida, U. humboldtii, U. praelonga, U. triflora* and *U. uliginosa* formed another cluster.

Key words: bladderwort, genetic diversity, ISSR, species relationship, terrestrial *Utricularia*

Development of different molecular markers based on polymerase chain reaction (PCR) offered the great opportunity to study genome organization, mating system and assessment of genetic diversity in different plant groups. The choice of a molecular marker depends on some factors like its reproducibility and simplicity. The markers that have high reproducibility and low cost are considered as the best markers for molecular studies and genome mapping. Recently, a novel molecular marker named inter simple sequence repeat (ISSR) was developed and has been found efficient for genetic diversity studies (Zietkiewicz *et al.* 1994). ISSR markers involve PCR amplification of DNA using a single primer consisted of a microsatellite (SSR) sequence such as $(GACA)_4$ or $(CA)_8$. Such sequences are common in the genome and are good target for fingerprinting techniques (Tautz & Renz 1984). ISSR primers are 15-mer to 22-mer and many of them consist of di-, tri-, tetra-, and pentanucleotide repeat motifs of which dinucleotide repeats are with anchor. The primers are generally anchored on the 3' or 5' end of the microsatellite motif. The advantages of ISSR markers include its simplicity, the potential for fewer template primer mismatch artifacts, higher levels of polymorphism and reproducibility, small amount of DNA used, no prior need for DNA sequence information, small reaction volumes for PCR and banding patterns can be easily scored (Wolfe *et al.* 1998). In several studies ISSR markers are reported to have advantages over other DNA markers such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) (Salimath *et*

al. 1995; Parsons *et al.* 1997; Pujar *et al.* 2002). Furthermore, ISSR overcomes many of the limitations of RAPD and RFLP (Tsumura *et al.* 1996).

ISSR markers have been used for DNA fingerprinting and assessment of genetic diversity in several economically meritorious, cultivated plant groups (Blair *et al.* 1999; Fernandez *et al.* 2002; Prevost & Wilkinson 1999; Huang & Sun 2000; Fang & Roose 1997). Recent studies have demonstrated the potential of these markers to detect genetic variation in *Spartina anglica* (Ayres & Strong 2001), *Botrichium pumicola* (Camacho & Liston 2001), *Lactoris fernandeziana* (Crawford *et al.* 2001), *Eurya nitida* (Deshpande *et al.* 2001), *Psammochloa villosa* (Li & Ge 2001) and *Humulus lupulus* (Patzak 2001). ISSR markers have also been found useful for species relationships in *Pandorea* (Jain *et al.* 1999), *Hyobanche* (Wolfe & Randle 2001), *Amaranthus* (Xu & Sun 2001) and *Nothofagus* (Mattioni *et al.* 2002).

Utricularia, the Lentibulariaceae, is distinct with carnivorous bladders, 2-lipped calyx, personate corolla and no true root. Several authors paid an attention on the morphology, cytology, physiology and palynology of this genus: Subramanyum (1979), Taylor (1989) and Crow (1992) contributed some of the important morphological account of this genus. Kondo (1966, 1973) and Casper & Manitz (1975) were the pioneers for chromosomes counting in different species of *Utricularia*. Recently, Rahman *et al.* (2001) reported new chromosome numbers from aquatic *Utricularia*. Thanikaimoni (1966), Huynh (1968) and Sohma (1975a, b) investigated pollen morphology of the genus. Sasago & Sibaoka (1985) made a physiological study on *U. vulgaris* and Thurston & Seabury (1975) performed a scanning electron microscopic work on *U. biflora*. Very recently, Müller *et al.* (2002) presented a general information on the evolution of the Lentibulariaceae based on molecular, morphological and physiological investigation. However, their study did not provide systematic relationships among the members of *Utricularia*.

Some species of *Utricularia* have similar vegetative morphology mainly with flower and leaf characters. A curious feature of *Utricularia* is that some of the morphologically similar species have been placed in different sections and in contrast, some other morphologically quite distinct species have been placed in the same section. Consequently, there has been an interest to study these species and there is a need to clarify the distance relationships among these species. The main focus of this study was to detect the genetic distance and similarity among the closely related species using ISSR approach and to elucidate relationship among them. Whether or not ISSR technique promises to distinguish the closely related species of *Utricularia* should be justified.

Materials and Methods

Plant materials

Ten terrestrial species, namely, *Utricularia alpina* Jacq., *U. amethystina* Salzm. ex A. St. Hil. & Girard, *U. bifida* L., *U. caerulea* L., *U. calycifida* Benj., *U. humboldtii* Schomb., *U. longifolia* Gardn., *U. praelonga* A. St. Hil. & Girard, *U. triflora* P. Taylor and *U. uliginosa* Vahl, were chosen for this study to assess the suitability of ISSR approach in the genus Utricularia. Plants were grown in axenic culture in Gamborg's B5 medium (Gamborg *et al.* 1968) (ten strength) at the Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Japan because the wild plants of the genus often contain other organisms in bladders and sometimes some algae and mycorrhizal fungi are also found in the plants.

DNA isolation

DNA was isolated from the leaves following Kawahara *et al.* (1995) with slight modification. 1.0-1.8g of leaf was homogenized in a mortar using

liquid nitrogen. The powdered tissue was transferred to a 20ml capacity of capped, sterilized centrifuged tube containing 10ml of wash buffer [0.1M Tris-HCl at pH 8.0, 2% 2-Mercaptoethanol, 1% Polyvinylpyrolidone K-30, 0.05M L-Ascorbic acid, dissolved in distilled water]. After shaking gently for 10 min the tube was centrifuged at 10,000 rpm at 20°C for 10 min. The supernatant was discarded from the tube and this process was repeated until the solution becomes transparent. After removing the supernatant, 10ml of CTAB buffer [2% Cetyltrimethylammonium bromide (CTAB), 1.4M NaCl, 0.1% Tris-HCl at pH 8.0, 20mM EDTA-Na_2, dissolved in distilled water] and 0.5ml 2-Mercaptoethanol were added to the tube followed by an incubation at 55°C for 60-90 min in order to supply the stabilization of DNA. Following that 10ml chloroform:isoamylalcohol (24:1) was added to the tube and was shaken gently for 10 min and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a new sterilized centrifuged tube and it was continued until there was no precipitation on the border of the supernatant layer and chloroform:isoamylalcohol layer. The final supernatant was transferred to an another centrifuged tube and 10ml of 2-propanol was added followed by a centrifugation at 10,000 rpm for 15 min at 4°C. After discarding the solution, 5ml 70% chilled ethanol was added to wash the pellet and was centrifuged at 10,000 rpm for 5 min. DNA was dried after decanting the ethanol and the dried DNA pellet was dissolved in 450µl TE solution (10mM Tris-HCl and 1mM EDTA) with 0.1mg/ml RNase (Sigma). After incubation for 1 hour at 37°C the solution was transferred in a 1.5ml sterilized ependorf tube. 250µl of neutral equilibrated phenol and 250µl of chloroform:isoamylalcohol (24:1) were added to the tube. The tube was centrifuged for 10 min after shaking for 10 min. The upper phase was transferred to a new tube. 500µl chloroform:isoamyl alcohol was added and was centrifuged for 10 min. The upper aquous solution was transferred to a new tube. Then 50µl of 3M sodium acetate and 500µl of 99.5% chilled ethanol were added to the tube and they were kept at –80°C for 20 min. The DNA pellet was found by spinning in a microcentrifuge at 15,000 rpm for 15 min at 4°C. The supernatant was removed and the pellet was washed with 70% chilled ethanol using centrifugation at 15,000 rpm for 15 min at 4°C. The ethanol was discarded and the DNA was dried into a Halogen Vacuum Concentrator for 3-5 min. The isolated DNA was dissolved in TE buffer and stored at –20°C.

Primer selection, PCR amplification and electrophoresis

We purchased a set of one hundred ISSR primers (primer set # 9) from the University of British Columbia, Biotechnology Laboratory (UBC, Vancouver, Canada). A total of 72 primers were tested for PCR amplification and finally we choose six primers, by their number and consistency of amplified fragments for analyzing *Utricularia* species. Primers used in this study along with their sequence are listed in Table 1. In PCR reaction nuclear DNA was used as template. We used 10µl reaction mixture for PCR and each 10µl amplification consisted of 10 ng of template DNA (nuclear DNA), 1µM of a single primer (UBC, Vancouver, Canada), 1µl of X10 Taq buffer, 0.8µl of dNTP mixture and 0.05µl of Taq polymerase enzyme. The reaction mixture was overlaid with 30µl of mineral

TABLE 1. ISSR primers used for genetic analysis of ten terrestrial *Utricularia* species

Primer names	Repeat	Anchor[a]	Annealing temperature (°C)
UBC842	$(GA)_8$	YG	50
UBC864	$(ATG)_6$	-	50
UBC888	$(CA)_7$	BDB	50
UBC889	$(AC)_7$	DBD	50
UBC890	$(GT)_7$	VHV	50
UBC891	$(TG)_7$	HVH	50

a: Y stands for pyrimidine, B for non-A, D for non-C, V for non-T and H for non-G residues

oil. After an initial denaturation at 94°C for 5 min, each cycle comprised 1 min denaturation at 94°C, 45 sec annealing at 50°C and 2 min extension at 72°C for 35 cycles and 5 min at 72°C for a final extension step. All amplifications were done in a PTC-100 thermal cycler. Amplified products were mixed with bromophenol dye and were analyzed on 1.5% agarose gels in 1XTAE buffer. Gels were run until a bromophenol blue indicator dye ran more than 70% from the well. After staining the gels in ethidium bromide for 30 min the bands were observed under UV light and photographed.

Data analysis

In order to estimate the genetic diversity and interspecific relationships among the ten species of *Utricularia*, we analyzed the banding patterns. ISSR bands were recorded in a binary data matrix scored as presence (1) or absence (0). The data matrices were analyzed using NTSYSpc software, version 2.1 (Rohlf 2000). Hamann's (1961) diversity index was used to estimate the similarity levels between the species studied. Pairwise genetic distance was calculated using the Jukes & Cantor (1969) distance coefficient. Principal coordinate analysis (PCoA) was performed in order to more effectively view the clustering pattern of the taxa included in this study.

Results and Discussion

ISSR survey

A total of 236 ISSR fragments were generated by six primers from ten species of *Utricularia*. The highest number of fragments was detected in *U. longifolia* (32) whereas the least number was found in *U. amethystina, U. humboldtii* and *U. uliginosa* containing 18 fragments each. In other species the number of fragments ranged from 20 in *U. bifida* to 31 in *U. triflora*. Primer UBC 889 presents the highest number of bands followed by the primer UBC 890 in all species. In contrast, primer UBC 888 and UBC 891 both generated the least number of bands (Fig. 1). The average number of fragments generated by all analyzed primers was 39.3.

Genetic diversity and species relationships

The potentiality of ISSR marker has been observed for genetic analysis in *Utricularia*. In order to determine genetic diversity among ten terrestrial *Utricularia* species we employed the ISSR technique and we found that Utricularia species could be characterized by ISSR markers. Across the ten species the estimated similarity index varied from 1.65 (*U. caerulea / U. longifolia*) to 7.32 (*U. alpina / U. longifolia*) indicating that *U. alpina* was most closely related to *U. longifolia* (Table 2). *U. bifida* exhibited high affinity with humboldtii whereas *U. amethystina* was closely related to *U. calycifida*. In other species this similarity index ranged from 2.12 (*U. calycifida / U. longifolia*) to 4.80 (*U. praelonga / U. uliginosa*). A pairwise genetic distance was calculated to know the distance relationships between the species. The highest genetic distance was observed between *U. caerulea* and *U. longifolia* (6.1) showing that these two species were most distantly related to each other while the lowest distance was detected between *U. alpina* and *U. longi-*

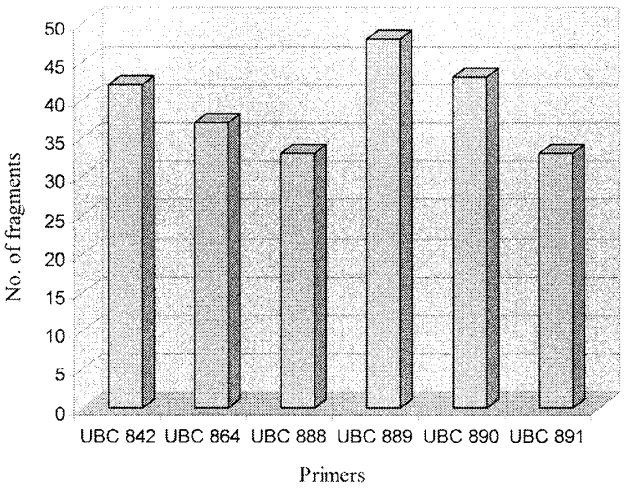

FIG. 1. Bar diagram showing the number of ISSR fragments generated by UBC primers across the ten species of *Utricularia*.

Table 2 Interspecific similarity index between *Utricularia* species based on ISSR data (Hamann's coefficient)

Species	*U. alpina*	*U. amethystina*	*U. bifida*	*U.caerulea*	*U. calycifida*	*U. humboldtii*	*U. longifolia*	*U. praelpnga*	*U. triflora*
U. amethystina	4.17								
U. bifida	3.22	4.33							
U. caerulea	2.44	4.17	4.17						
U. calycifida	2.28	4.96	4.33	3.22					
U. humboldtii	3.54	3.07	5.27	4.48	4.64				
U. longifolia	7.32	3.07	2.75	1.65	2.12	3.7			
U. praelpnga	3.07	4.17	4.17	3.7	3.22	4.17	2.59		
U. triflora	3.07	4.17	3.22	3.7	3.85	3.85	2.28	2.44	
U. uliginosa	3.54	4.33	4.01	3.22	4.33	4.33	3.07	4.8	2.59

Table 3 Pairwise genetic distance between *Utricularia* species based on ISSR data (Jukes & Cantor coefficient)

Species	*U. alpina*	*U. amethystina*	*U. bifida*	*U.caerulea*	*U. calycifida*	*U. humboldtii*	*U. longifolia*	*U. praelpnga*	*U. triflora*
U. amethystina	3.69								
U. bifida	4.5	3.56							
U. caerulea	5.26	3.69	3.69						
U. calycifida	5.42	3.07	3.56	4.5					
U. humboldtii	4.22	3.07	2.84	3.44	3.31				
U. longifolia	1.47	4.65	4.95	6.1	5.58	4.08			
U. praelpnga	4.65	3.69	3.69	4.08	4.5	3.69	5.1		
U. triflora	4.65	4.5	4.5	4.08	3.95	3.95	5.42	5.26	
U. uliginosa	4.22	3.82	3.82	4.5	3.56	3.56	4.65	3.19	5.1

folia (1.47) (Table 3). High distance relationship was also observed between *U. calycifida* and *U. longifolia* (5.58); *U. alpina* and *U. calycifida* (5.42) and *U. longifolia* and *U. triflora* (5.42). The distance in other species varied from 2.84 (*U.bifida / U. humboldtii*) to 5.26 (*U. alpina / U. caerulea; U. praelonga / U. triflora*).

Principal Coordinate Analysis (PCoA) was conducted to view the clustering pattern and species relationships in *Utricularia*. The PCoA analysis of ISSR data resulted in placing *U. alpina* and *U. longifolia* together and the other species *U. amethystina, U. bifida, U. caerulea, U. calycifida, U. humboldtii, U. praelonga, U. triflora* and *U. uliginosa* clustered together (Fig. 2).

Recently, Araki (2000) conducted a molecular study in *Utriculria australis* based on isozyme analysis. Alcohol dehydrogenase (ADH) and phosphoglucoisomerase (PGI) separated *U. australis* to f. *australis* and f. *tenuicaulis* suggesting that these taxa are genetically distinct. In his study, alcohol dehydrogenase (ADH) was found more efficient than phosphoglucoisomerase (PGI) to detect the genetic variation among these infraspecies taxa. Rahman & Kondo (2002) first applied the ISSR technique in *Utricularia* and their study presented good interspecific relationships that led us to undertake this present study. The present study has demonstrated that ISSR markers are useful to detect genetic variation and species distance in *Utricularia*. Molecular analysis of ISSR data in *Utricularia* resolved the species into two clusters: one cluster constituted from *U. alpina* and *U. longifolia* and the other consisted of *U. amethystina, U. bifida, U. caerulea, U. calycifida, U. humboldtii, U. praelonga, U. triflora* and *U. uliginosa* (Fig. 2). *U. alpina*

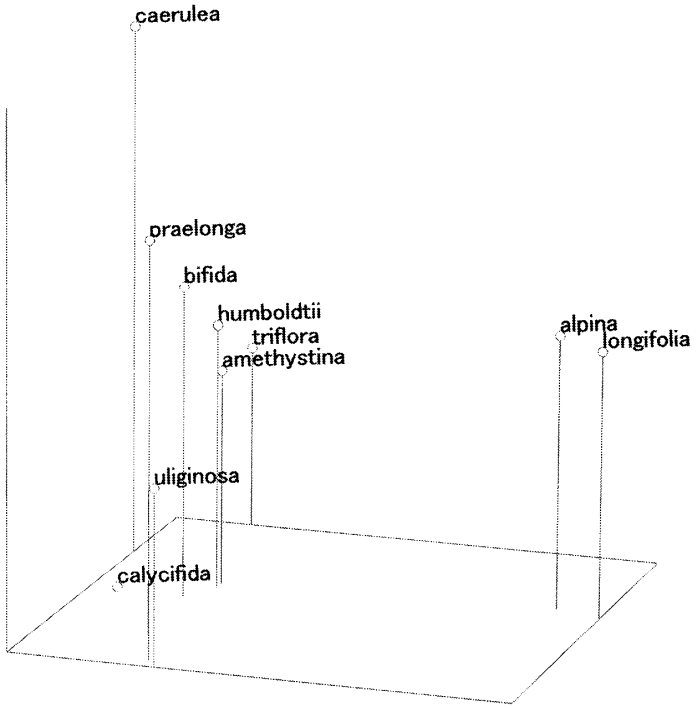

FIG. 2. Principal coordinate analysis for ten *Utricularia* species based on ISSR data.

and *U. longifolia* showed the highest affinity among the species studied. Morphologically these species are similar to each other by sharing elliptic or obovate leaves, ovate to ovate-deltoid calyx lobes, curved filament, and ovoid ovary, and high affinity was supported by our analysis. Although Taylor (1989) placed these two species in two different sections, our results suggested that these species might be placed very close to each other in the same section. However, further investigation using some other molecular tools is necessary to figure out whether or not these species should be placed in the same section. The other cluster resulted from PCoA analysis bearing eight species included *U. amethystina, U. bifida, U, caerulea, U. calycifida, U. humboldtii, U. praelonga, U. triflora* and *U. uliginosa*. *U. calycifida* is related to *U. amethystina* by having unequal calyx lobes, globose capsule and obliquely ovoid seeds but dissimilar from each other by trap and ovary characters. Our results showed that *U. calycifida* presented high affinity with *U. amethystina* indicating the close relationship to each other. Moreover, *U. calycifida* showed a high similarity with *U. humboldtii* (Table 2) whereas the former showed a high genetic distance with *U. longifolia* (Table 3). Some characteristics including basifixed bracts, linear or linear to subulate bracteoles, distinct anther thecae and subulate spur placed *U. bifida* and *U. humboldtii* in the same line and a high similarity between these species was obtained in our analysis. From the morphological point of view, *U. praelonga* and *U. uliginosa* show affinity with each other by possessing ovate bracts, subulate or narrowly linear bracteoles and short style and in our analysis these species were found close to each other as well.

Sohma (1975b) showed that *Utricularia bifida* is allied to *U. caerulea* by possessing tricolporate pollen. In our investigation these species fall in the same cluster as well (Fig. 2). Considering the cytology of *Utricularia*, chromosome numbers have been counted only in *U. alpina* ($2n = 18$; Kondo 1966 as *Orchyllium alpinum*), *U. caerulea* ($2n = 36$; Kondo 1973 as *U. racemosa*) and *U. uliginosa* ($2n =$

16; Tanaka & Uchiyama 1988 as *U. yakusimensis*) out of the species analyzed. Since the chromosomal information is very much lacking and fragmentary, we could not compare our results with cytological investigation properly.

ISSR banding patterns have been found correlated with the phenomenon of speciation. Wolfe *et al.* (1998) applied ISSR markers to assess the effectiveness of ISSR banding patterns for elucidating patterns of hybridization and diploid hybrid speciation in *Penstemon* in a hybrid complex including *P. centranthifoilus, P. clevelandii, P. grinnellii* and *P. spectabilis*. Their results showed that *P. clevelandii* is a diploid hybrid species derived from *P. centranthifoilus* and *P. spectabilis* and supported the previous hypothesis in this hybrid complex based on morphological and artificial crossing studies. Wolfe & Elisens (1993, 1994, 1995) studied this hybrid complex using allozymes, restriction site variations of nuclear rDNA and chloroplast DNA but these studies failed to support for diploid hybrid speciation.

In ISSR analysis the data are usually analyzed in the same way as RAPD markers. There are some inherent difficulties with RAPD associated with their reproducibility, dominant nature and uncertain marker homology (Weising *et al.* 1995). Like RAPD markers, ISSR technique has also some limitations although it has gained acceptance as a useful approach in a wide range of cultivated and wild plants. Limitations encountered in ISSR markers includes: bands are scored as dominant markers, genetic diversity estimates are based on diallelic characters, clean DNA template is needed and optimization of initial reactions is required (Wolfe *et al.* 1998). Even with these few limitations, ISSR markers provide an attractive alternative to RAPD markers and can be implemented much more easily than amplified fragment length polymorphisms (AFLP; Wolfe & Liston 1998).

Finally, in our experiment ISSR approach was found useful at determining the genetic diversity and species relationship in *Utricularia*. Therefore, we will apply this novel technique for further research aimed at determining the genetic distance and systematic relationships among other species of the genus *Utricularia*.

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